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Original Paper

Resveratrol Protects Murine Chondrogenic ATDC5 Cells Against LPS-Induced Inflammatory Injury Through Up-Regulating MiR-146b

Hui Jin[a] Hong Zhang[b] Tingjian Ma[a] Hongjia Lan[a] Shibin Feng[a] Haiyan Zhu[a] Youbo Ji[a]

[a]Department of Orthopedic, The Second Hospital of Jilin University, Changchun, [b]Department of Clinical Medicine, Changchun Medical College, Changchun, China

Key Words
Resveratrol • Osteoarthritis • ATDC5 cell • LPS • MiR-146b

Abstract
Background/Aims: Resveratrol (RSV) has been reported as a promising oral supplementation for osteoarthritis treatment, while the mechanism of its action is still unclear. The specific aim of this study is to decode one of the mechanisms by which RSV protects chondrocyte. ***Methods:*** Mouse chondrogenic cell line ATDC5 was treated with 30 μM RSV for 24 h, and 10 μg/ml LPS for 12 h, after which cell viability, apoptosis, and the release of pro-inflammatory cytokines were assessed. The expression of miR-146b in ATDC5 cells was silenced by the specific inhibitor transfection, and then cell viability, apoptosis and inflammation were re-assessed. ***Results:*** The IC50 value of LPS in ATDC5 cells was about 10.27 μg/ml. LPS with a dosage of 10 μg/ml repressed cell viability, induced apoptosis, and increased the release of IL-1β, IL-6 and TNF-α. RSV pre-treatment (30 μM) significantly alleviated LPS-induced apoptosis and inflammation. More importantly, miR-146b was up-regulated by RSV, and the protective functions of RSV on ATDC5 cells were attenuated by miR-146b silence. Further, NF-κB and p38MAPK pathways were activated by LPS, and were deactivated by RSV. Besides, RSV-induced the deactivation of NF-κB and p38MAPK pathways was reversed by miR-146b silence. ***Conclusions:*** Our findings suggest that RSV protects ATDC5 cells from LPS-induced inflammatory and apoptotic injury via up-regulation of miR-146b and thereby deactivation of NF-κB and p38MAPK pathways.

Introduction

Osteoarthritis, a degenerative joint disease commonly affects the knee, is a leading cause of disability worldwide [1]. Joint inflammation, cartilage breakdown and bone remodeling contribute to a syndrome of chronic pain, stiffness and impaired movement

Youbo Ji
Department of Orthopedic, The Second Hospital of Jilin University
No. 218, Ziqiang Street, Changchun, Jilin 130041 (China)
Tel. +86-431-81136747, Fax +86-431-81136747, E-Mail youbo1226@sohu.com

[2]. Recent years, the prevalence of osteoarthritis is significantly increased with the rapid increase of aging population and the rising obesity. According to patient's needs, anatomical distribution, disease phase and progression, as well as comorbidities, osteoarthritis patients are always managed by supportive nonpharmacological therapy, systemic pharmacological therapy, localized intra-articular therapy, and surgery [2]. However, an ongoing treatment gap for patients with osteoarthritis is still remaining [3, 4], which calls for effective treatment options.

Resveratrol (RSV), 3, 4',5-trihydroxy-trans-stilbene, is a polyphenolic phytoalexin that is produced by a wide variety of plants, berries, and fruits, and is mainly found in grape skin and red wine [5]. Numerous studies have reported RSV as an anti-oxidative, anti-inflammatory, anti-apoptotic, chemoprotective, anti-diabetic, anti-cancer, and anti-viral agent [6-11]. *In vitro* evidences have demonstrated that RSV suppressed IL-1β-induced inflammatory signaling and apoptosis in human chondrocytes, implying the potential of RSV for use in the treatment of osteoarthritis [12, 13]. In a rat model of osteoarthritis, RSV significantly inhibited the induction of clinical scores in rats with osteoarthritis [14]. Another *in vivo* study mentioned that RSV prevented the progression of osteoarthritis by decreasing chondrocyte apoptosis [15]. The anti-inflammatory and anti-apoptotic effects of RSV in these studies were observed, and suggested RSV as a promising oral supplementation for osteoarthritis treatment. But, to date, the mechanisms underlying RSV's protective functions towards chondrocyte are still unclear and need to be decoded.

microRNAs (miRNAs) are a group of short, non-coding RNAs. miRNAs were previously recognized as "noise DNA", but recent studies evidenced them as key regulators in a wide range of biochemical pathways, including cell proliferation, apoptosis, differentiation, and autophagy. Aberrant miRNA levels have been observed in osteoarthritis [16], and some of them have been considered as therapeutic targets, as delivery of miRNA can modify the process of osteoarthritis [17]. miR-146b is known as a post-transcriptional gene silencer, which is critical in the control of immune response [18]. It has been previously shown that miR-146b was up-regulated in chondrocyte which were isolated from osteoarthritis articular cartilage, suggesting miR-146b as an anti-inflammatory mediator in chondrocyte [19]. The specific aim of this study is to explore whether miR-146b is implicated in the protective functions of RSV on LPS-injured chondrogenic cells.

Materials and Methods

Cell culture and treatment

Mouse chondrogenic cell line ATDC5 was purchased from European Collection of Authenticated Cell Cultures (ECACC, Porton Down, Wiltshire, UK). The cells were cultured in DMEM: Ham's F-12 (1:1) medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM Glutamine (Sigma-Aldrich) and 5% foetal bovine serum (FBS, Gibco, Grand Island, NY, USA). The cells were maintained at 37°C in a humidified incubator with 5% CO_2. Subcultures were obtained after 2-3 days of culturing using trypsin/EDTA solution (Sigma-Aldrich). All cells were used between the fifth and tenth passages.

LPS from Escherichia coli O111:B4 was purchased from Sigma-Aldrich. LPS with concentrations ranged from 0 to 20 μg/ml were used to treat ATDC5 cells for 12 h to stimulate inflammatory injury.

RSV with purity greater than 99% was purchased from Sigma-Aldrich. RSV was dissolved in DMSO (⩾99.7%, Sigma-Aldrich) and made up with the medium so that the final concentration of the DMSO was less than 0.1%. 0.1% DMSO without RSV was used as a blank control. ATDC5 cells were treated by 0-50 μM RSV for 24 h before LPS treatment.

Cell viability assay

ATDC5 cells were seeded in 96-well plates with a density of 5000 cells/well. After adherence, the culture medium was replaced by fresh medium containing 30 μM RSV. The plates were incubated at 37°C for 24 h, followed by 12 h treatment with 10 μg/ml LPS. After treatment, the cells in each group were washed twice with PBS and then 20 μl Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg,

MD) was added into each well and incubated for another 4 h at 37°C. The absorbance was measured using a Microplate Reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm.

Apoptosis assay

Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China) was used to detect the apoptotic cells. ATDC5 cells were seeded in 6-well plates with a density of 5×10^5 cells/well. When the cells were grown to about 80%~90% confluence, RSV and LPS were added simultaneously or respectively. After treatment, the cells were collected and resuspended in 200 μl Binding Buffer (1×10^5 cells/sample) containing 10 μl Annexin V-FITC and 5 μl PI. The adherent and floating cells were combined. The samples were incubated for 30 min in the dark on ice, and then 300 μl PBS was added. Flow cytometer (Beckman Coulter, USA) was used to discriminate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V- and PI-positive).

ELISA

ATDC5 cells were grown to about 80%~90% confluence in 6-well plates. After treatment with RSV and LPS simultaneously or respectively, the culture supernatant of ATDC5 cells was collected for use in the detection of inflammatory cytokine concentrations. Mouse IL-1β (Cat. No.: CSB-E08054m), mouse IL-6 (Cat. No.: CSB-E04639m), and mouse TNF-α (Cat. No.: CSB-EQ023955MO) ELISA kits (Cusabio, Wuhan, China) were conducted according to the manufacturer's instructions. Optical density was detected at 450 nm using the iMark microplate reader (Bio-Rad, Hercules, CA, USA).

miRNA transfection

For miR-146b silencing, the specific inhibitor for mmu-miR-146b with sequence of AGCCUAUGGAAUUCAGUUCUCA was purchased from GenePharma Co. (Shanghai, China). A scrambled miRNA was transfected as a negative control (NC). Transfection was performed under antibiotic-free in 6-well plates by using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA). The final concentration of miR-146b inhibitor (anti-miR-146b) and NC used in the transfection was 100 nM. At 48 h of transfection, cells were collected for use in the following experiments.

qRT-PCR

To analyze the mRNA level expressions of IL-1β, IL-6 and TNF-α, total RNA was extracted from ATDC5 cells which were grown in 24-well plates by using TRIzol reagent (Invitrogen). Five micrograms of total RNA of each sample were subjected to reverse transcription by using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). FastStart Universal SYBR Green Master (Roche) was conducted for qRT-PCR. The levels of IL-1β, IL-6 and TNF-α were normalized to β-actin. To analyze the RNA level of miR-146b, total miRNAs in ATDC5 cells were isolated by using miRNeasy Mini Kit (Qiagen, Shenzhen, China). Reverse transcription was performed by using the Mir-X™ miRNA First-Strand Synthesis Kit and qRT-PCR was performed by Mir-X™ miRNA qRT-PCR SYBR® Kit (both from Takara, Dalian, China). U6 was used as a reference control for normalizing miR-146b expression. Fold changes were calculated by the classic $2^{-\Delta\Delta Ct}$ method. The primary sequences were listed in Table 1.

Table 1. The primary sequences used in qRT-PCR analysis

Gene	Sequences
IL-1β	forward, 5'-GCAACTGTTCCTGAACTCAACT-3';
	reverse, 5'-ATCTTTTGGGGTCCGTCAACT-3'
IL-6	forward, 5'-TCCTACCCCAATTTCCAATGC-3';
	reverse, 5'-CCACAGTGAGGAATGTCCACAA-3'
TNF-α	forward, 5'-TCTTCCCTGAGGTGCAATGC-3';
	reverse 5'-GCTCCGTTTTCACAGAAAACATG-3'
miR-146b	forward, 5'-GGGTGAGAACTGAATTCCA-3';
	reverse 5'-CAGTGCGTGTCGTGGAGT-3'
U6	forward, 5'-CTCGCTTCGGCAGCACATATACT-3';
	reverse, 5'-ACGCTTCACGAATTTGCGTGTC-3'
β-actin	forward, 5'-GGCTGTATTCCCCTCCATCG-3';
	reverse, 5'-CCAGTTGGTAACAATGCCATGT-3'

Western blot

Total protein in ATDC5 cells which were grown in 24-well plates was isolated by cell lysis buffer (20 mM Tris-HCl (pH7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% $C_{24}H_{39}O_4Na$, 1% SDS and 1 mM PMSF) for 30 min over ice. The concentration and purity were detected by the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The whole-cell extracts were resolved over SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature, followed by incubation with the specific primary antibodies for the detection of pro

caspase-3 (ab90437), cleaved caspase-3 (ab13847), TNF-α (ab1793), p-p65 (ab86299), p65 (ab32536), p-IκBα (ab133462), IκBα (ab7217), p-p38MAPK (ab47363), p38MAPK (ab170099), β-actin (ab8226) (Abcam, Cambridge, MA), Bcl-2 (sc-509), Bax (sc-20067), IL-1β (ab156791), and IL-6 (sc-57315) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were probed by the secondary antibodies for 1 h at room temperature, after which the blots were visualized by enhanced chemiluminescence (ECL) method. The intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All experiments were done in triplicate. Data represented as mean ± SD. Statistical differences between two or more groups were analyzed on SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA) by using one-way analysis of variance (ANOVA) with Duncan procedure. A p-value of < 0.05 was considered as a significant difference.

Results

LPS induces apoptotic and inflammatory injury in ATDC5 cells

To begin with, ATDC5 cells were subjected to an increasing dosage of LPS for 12 h. As a result, viability of ATDC5 cells was significantly reduced by LPS ($p < 0.05$), the IC50 value of LPS toward ATDC5 cells was about 10.27 μg/ml (Fig. 1A). Therefore, 10 μg/ml was selected as a LPS-stimulating condition for use in the following experiments. Fig. 1B and 1C indicated that LPS induced a significant increase in apoptotic cell rate ($p < 0.05$), remarkable up-regulations of cleaved caspase-3 and Bax, and a down-regulation of Bcl-2. Moreover, the protein and mRNA levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in cell as well as their concentrations in cell culture supernatant were all increased notably by LPS stimulation ($p < 0.05$, Fig. 1D-1F).

RSV protects ATDC5 cells against LPS induced injury

RSV with dosage of 0-50 μM was used to treat ATDC5 cells for 24 h, after which cell viability was monitored by CCK-8 assay. Results in Fig. 2A showed that, 10, 20 and 30 μM of RSV did not significantly affect viability of ATDC5 cells. But, higher dosages (40 and 50 μM) of RSV induced significant increases in cell viability ($p < 0.05$). To see the effects of RSV on LPS-injured ATDC5 cells, 30 μM was selected as a RSV-treating condition for the further functional analyses. As results shown in Fig. 2B, RSV pre-treatment significantly improved the viability of LPS-treated cells from 51.6% to 83.0% ($p < 0.05$). LPS-induced apoptosis was reduced by RSV pre-treatment, as the apoptotic cell rate was decreased from 14.1% to 8.0% ($p < 0.05$), protein levels of cleaved caspase-3 and Bax were decreased, and protein level of Bcl-2 was increased (Fig. 2C and 2D). It is not surprising that, LPS-induced up-regulation and the release of IL-1β, IL-6 and TNF-α were all alleviated by RSV pre-treatment ($p < 0.05$, Fig. 2E-2G).

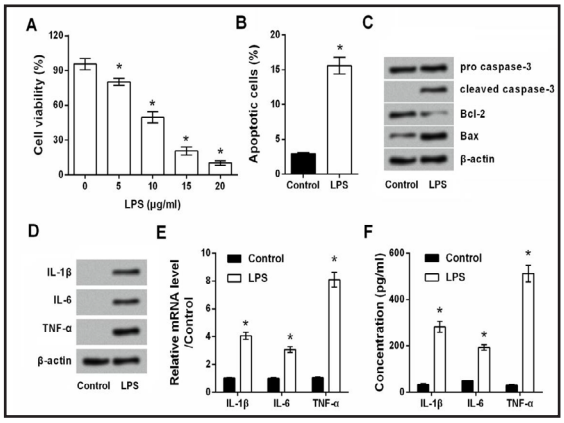

Fig. 1. Effects of LPS on ATDC5 cells. (A) ATDC5 cells were subjected to an increasing dosage of LPS for 12 h, after which cell viability was monitored. (B) Apoptotic cell rate, (C) expression levels of apoptosis-related proteins, (D) protein levels of pro-inflammatory cytokines, (E) mRNA levels of pro-inflammatory cytokines, and (F) concentrations of pro-inflammatory cytokines in the culture supernatant were assessed, after ATDC5 cells were treated with or without 10 μg/ml LPS. * p<0.05 compared to control group (the cell without LPS treatment).

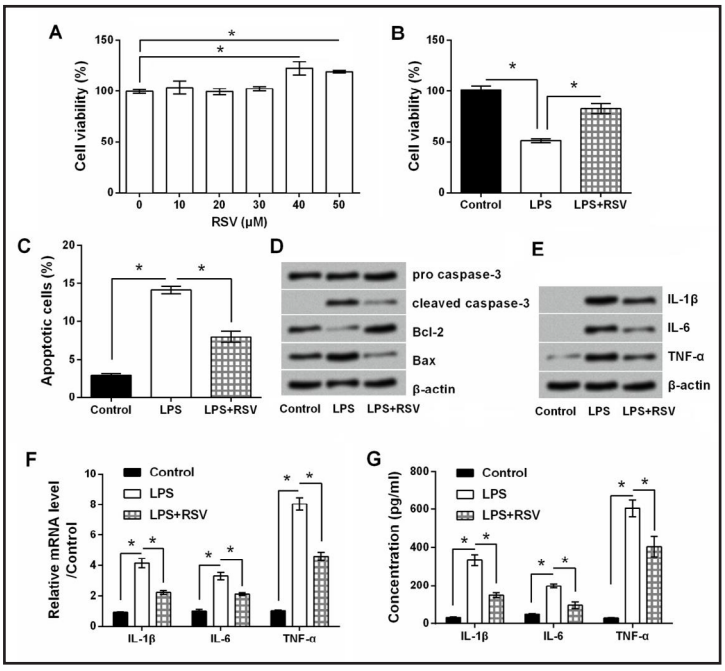

Fig. 2. Effects of RSV on LPS-injured ATDC5 cells. (A) ATDC5 cells were subjected to an increasing dosage of RSV for 24 h, after which cell viability was monitored. (B) Cell viability, (C) Apoptotic cell rate, (D) expression levels of apoptosis-related proteins, (E) protein levels of pro-inflammatory cytokines, (F) mRNA levels of pro-inflammatory cytokines, and (G) concentrations of pro-inflammatory cytokines in the culture supernatant were assessed, after ATDC5 cells were in turn treated with 30 μM RSV for 24 h, and 10 μg/ml LPS for 12 h. * $p<0.05$ compared to the indicated group.

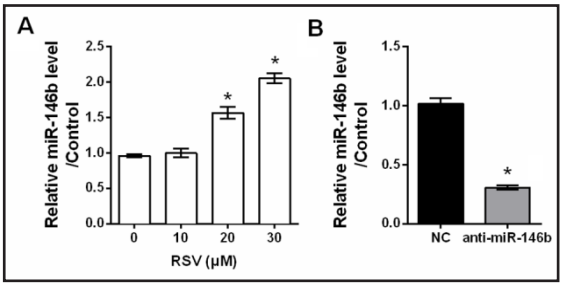

Fig. 3. Effects of RSV on the expression of miR-146b. (A) RNA levels of miR-146b in ATDC5 cells were detected after treating with an increasing dosage of RSV for 24 h. * $p<0.05$ compared to the 0 μM RSV group. (B) RNA levels of miR-146b in ATDC5 cells were detected after transfection with miR-146b inhibitor (anti-miR-146b) and its scrambled control (NC). * $p<0.05$ compared to the NC group.

RSV promotes the expression of miR-146b

Considering the importance of miR-146b in osteoarthritis, the regulatory impacts of RSV on miR-146b expression levels were explored. qRT-PCR data showed that 20 and 30 μM of RSV resulted in significant increases in the RNA levels of miR-146b ($p < 0.05$, Fig. 3A). These data indicated that miR-146b might be involved in the protective functions of RSV in LPS-injured ATDC5 cells. To verify this hypothesis, the expression of miR-146b in ATDC5 cells was silenced by transfection with the specific inhibitor of mmu-miR-146b. Compared to the NC group, anti-miR-146b significantly decreased miR-146b expression ($p < 0.05$, Fig. 3B).

RSV protects ATDC5 cells against LPS induced injury via up-regulation of miR-146b

The miR-146b-silenced cells obtained from transfection were in turn treated with RSV and LPS. The increase of cell viability, the decrease of apoptotic cell rate, the down-regulations of cleaved caspase-3 and Bax, as well as the up-regulation of Bcl-2 induced by RSV were attenuated or even abolished by anti-miR-146b transfection ($p < 0.05$, Fig. 4A-4C). Also, the reduced expression and release of IL-1β, IL-6 and TNF-α by RSV were reversed by anti-miR-146b transfection ($p < 0.05$, Fig. 4D-4F).

RSV inactivates NF-κB and p38MAPK pathways via up-regulation of miR-146b

Western blot analysis was performed to detect the expression changes of core proteins in NF-κB and p38MAPK pathways. As shown in Fig. 5A and 5B, p65, IκBα and p38MAPK were

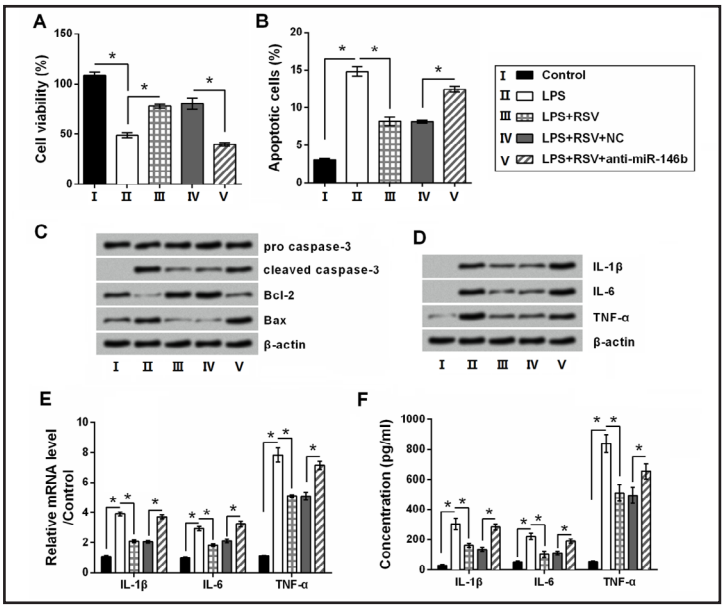

Fig. 4. Effects of miR-146b on the protective functions of RSV in ATDC5 cells. (A) Cell viability, (B) Apoptotic cell rate, (C) expression levels of apoptosis-related proteins, (D) protein levels of pro-inflammatory cytokines, (E) mRNA levels of pro-inflammatory cytokines, and (F) concentrations of pro-inflammatory cytokines in the culture supernatant were assessed, after ATDC5 cells were in turn transfected with anti-miR-146b or NC, treated with 30 μM RSV for 24 h, and treated with 10 μg/ml LPS for 12 h. * p<0.05 compared to the indicated group.

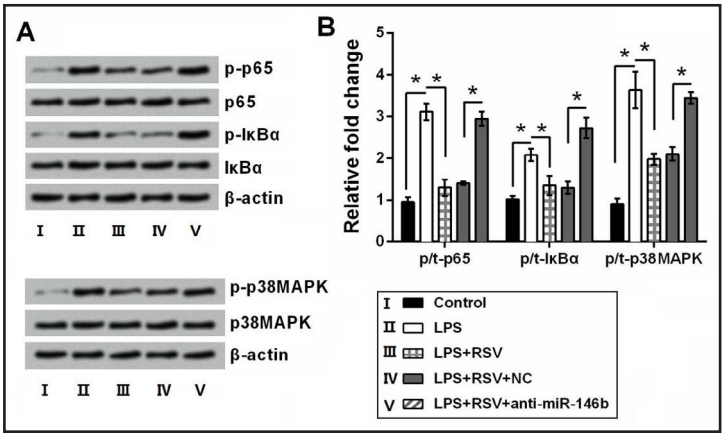

Fig. 5. Effects of RSV on the activation of NF-κB and p38MAPK pathways. (A) Protein levels of core proteins in NF-κB and p38MAPK pathways were detected, after ATDC5 cells were in turn transfected with anti-miR-146b or NC, treated with 30 μM RSV for 24 h, and treated with 10 μg/ml LPS for 12 h. (B) Quantitative analyses based on the intensity of protein bands. * p<0.05 compared to the indicated group.

significantly phosphorylated in response to LPS ($p < 0.05$). LPS-induced phosphorylation of p65, IκBα and p38MAPK were alleviated by RSV pre-treatment, while RSV could not alleviate the phosphorylation when anti-miR-146b was transfected into cell ($p < 0.05$).

Discussion

Osteoarthritis is a chronic degenerative joint disease, burdening patients worldwide [1]. The pathophysiology of osteoarthritis is still unclear, but evidences have proved that pro-inflammatory cytokines produced from cartilage are released into synovial space and participate in the breakdown of articular cartilage, collagen fibers, ligaments and menisci [20, 21]. Herein, ATDC5 cells were subjected to LPS to induce an *in vitro* model of cartilage damage. The IC50 value of LPS toward ATDC5 cells was about 10.27 μg/ml. Consistent with previous studies [22, 23], 10 μg/ml LPS resulted in a significant induction of apoptosis, and a significant release of pro-inflammatory cytokine, indicating LPS induced inflammatory and apoptotic injury in ATDC5 cells. More importantly, RSV could protect ATDC5 cells from LPS-induced cell damage, and miR-146b might be involved in these protective actions.

RSV is a polyphenolic compound found in several plants [5]. The natural function of RSV is to protect plants against fungal infection, while recent studies suggest it as a possible

therapeutic option in some organ-specific or systemic autoimmune diseases [24]. RSV is selectively toxic for a wide variety of tumor cells, but is helpful for the growth of a certain number of normal cells, such as endothelial cells, lymphocytes, and chondrocytes [25]. Here, we found that low dosage of RSV (0-30 μM) has no significant impact on ATDC5 cells viability, but high dosage of RSV (40 and 50 μM) promoted cell viability. A similar result was reported by Liu and his colleagues [26], suggesting RSV could promote the survival of ATDC5 cells. Then, 30 μM was selected as a RSV-pretreating condition for revealing the functions of RSV on LPS-injured ATDC5 cells. The reason for why we chose 30 μM is that we should exclude the survival promoting effects of high dosage RSV on cell. We demonstrated that RSV switched LPS-induced apoptosis (mitochondrial-dependent) into survival. Besides, RSV alleviated LPS-induced inflammation, as the release of IL-1β, IL-6 and TNF-α was significantly repressed by RSV preconditioning. Consistent with previous studies [14, 27, 28], these findings suggested the anti-apoptotic and anti-inflammatory functions of RSV on ATDC5 cells and provided evidences that RSV might be a promising therapeutic option for osteoarthritis treatment.

It is believed that RSV affects the initiation and progression of many diseases through several mechanisms. Although no mechanistic link between any particular miRNA and RSV has been identified, RSV effects depend at least in part upon the modification of the expression of inflammation-associated miRNAs, such as miR-663, miR-155, miR-663, and miR-21 [29]. It has been reported that the expression of miR-146b was elevated in response to cytokines or LPS [18]. miR-146b has been proposed to be an anti-inflammatory miRNA in chondrocyte [19], and an anti-apoptotic miRNA in dendritic cell and cardiomyocyte [30, 31]. Herein, we demonstrated that RSV preconditioning induced a significant increase of miR-146b expression, and the protective functions of RSV on ATDC5 cells against LPS were attenuated or even abolished by miR-146b silence. These data suggest that RSV protects ATDC5 cells against LPS-induced inflammatory and apoptotic injury via up-regulation of miR-146b.

In the developing stages of osteoarthritis, various signaling pathways are involved in, including NF-κB and p38MAPK pathways. p38MAPK can be strongly activated by pro-inflammatory cytokines, such as IL-1 and TNF-α, and p38MAPK is functional in the regulation of the inflammatory response [32]. NF-κB proteins include p65 (Rel A), Rel B, c-Rel, p50 (NF-κB1) and p52 (NF-κB2) [33]. In the cytoplasm of quiescent cells, NF-κB is inactivated by binding with IκBs (inhibitors of NF-κB); while under stimulation, a cascade of events was triggered to phosphorylate IκBs and activate NF-κB pathway [34]. The activation of NF-κB pathway promotes the transcription of pro-inflammatory genes, including IL-1β, IL-6, TNF-α and IL-8, and ultimately participates in inflammatory response [35]. There exists an interesting cross-talk between NF-κB and p38MAPK pathways, that the phosphorylated p38MAPK will promote the activation of NF-κB [36]. In the present study, we found that LPS-induced the activations of NF-κB and p38MAPK pathways were alleviated by RSV preconditioning, and the alleviating effects of RSV were abolished by miR-146b silence. These data indicate that NF-κB and p38MAPK pathways are involved in the protective functions of RSV on ATDC5 cells.

In conclusion, our findings provide evidence that RSV may be a promising therapeutic option for osteoarthritis treatment, as it protects ATDC5 cells from LPS-induced inflammatory and apoptotic injury. Besides, we for the first time suggest that RSV protects ATDC5 cells possibly by up-regulation of miR-146b and thereby deactivation of NF-κB and p38MAPK pathways.

Disclosure Statement

The authors declare to have no competing interests.

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